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IMMUNIZATION OF MICE WITH IRRADIATED *PASTEURELLA TULARENSIS*

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ABSTRACT

Viable attenuated tularemia vaccines have been shown to be effective in immunizing mice and other animals against challenge with highly virulent Pasteurella tularensis. Nonviable preparations, however, induced little or no resistance to challenge with even small numbers of virulent organisms. It seemed reasonable to expect that antigenicity would be retained to a greater extent if the organisms were killed by irradiation rather than by chemicals or heat.

Vaccines rendered nonviable by the action of X-radiation produced levels of immunity such that 20 to 30 per cent of immunized mice survived intraperitoneal challenge with moderate doses of the highly virulent SCHU S4 strain. Cysteamine at a concentration of 0.02 M was added to the bacterial suspension prior to irradiation to minimize the indirect effects of irradiation. Vaccines contained approximately 10^9 nonviable organisms per milliliter. Proof of nonviability of irradiated vaccines was based on (a) absence of colonies when irradiated vaccines were plated on solid medium, (b) failure to isolate P. tularensis from sacrificed immunized animals, and (c) the inability of treatment with streptomycin during immunization to interfere with the development of immunity.

I. INTRODUCTION

Immunization of susceptible animals with nonviable tularemia vaccines has failed to evoke levels of immunity such that the animals survive challenge with appreciable numbers of fully virulent organisms. Accordingly, it has been necessary to utilize less demanding criteria of protection for demonstration of the immunizing activity of such preparations.[1-5] Vaccines consisting of viable attenuated organisms, however, evoke appreciable immunity against challenge with fully virulent organisms even in susceptible animals such as white mice.[6] Evidently the agents employed to render vaccines nonviable destroy the protective antigenicity associated with viable organisms.

It seemed possible that this antigenicity might be retained to a greater extent if the organisms were killed by the action of ionizing radiation rather than by heat or chemicals. This supposition is based on the hypothesis that under conditions that inhibit indirect effects of irradiation, organisms are rendered incapable of reproduction primarily by damage to genetic material rather than by degradation of other structures of the cell.[7] Evidence had been obtained that irradiated vaccines were in fact more effective in immunization against Ehrlich's ascites carcinoma than were vaccines prepared in other ways.[8] This report describes experiments testing the antigenicity of Pasteurella tularensis rendered nonviable by exposure to ionizing radiation.

II. MATERIALS AND METHODS

A. STRAINS

The fully virulent SCHU S4 strain of P. tularensis was used for challenge. Vaccine preparations were usually derived from this strain. A strain of reduced virulence designated LVS[6] was used in some of the studies as indicated. Stock cultures were maintained at 5°C on SB agar[9] modified by the omission of glutamic acid and N-acetyl glucosamine. Twenty-four-hour cultures were employed for all vaccine and challenge preparations. The SCHU S4 strain was tested for virulence in rabbits and guinea pigs and was considered satisfactory for challenge if an intraperitoneal injection of ten or less organisms was fatal for these animals in less than ten days.*

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* In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

B. IRRADIATED VACCINE PREPARATION

The surface growth from a 24-hour agar culture was washed with 0.1 per cent gelatin in physiological saline. Unless stated otherwise, the concentration of bacteria in the suspending fluid was approximately 10^{10} organisms per milliliter. β-Mercaptoethylamine hydrochloride (cysteamine) was added at a final concentration of 0.02 M to the bacterial suspension prior to irradiation.

A General Electric Maxitron X-ray machine operating at one million electron volts and three milliamperes (no added filtration; half-value layer equal to 3.6 millimeters of lead) was used to irradiate 2.0 or 4.0 milliliters of bacterial suspension in 35-mm-diameter Carrel flasks. Four flasks were placed one on top of another in the nose cone of the X-ray machine. Irradiation was periodically interrupted to change the sequence of flasks. Consequently, the distance from the target to the bacterial suspension varied from 10 to 13 centimeters and the dose rate from 8700 roentgens to 5150 roentgens per minute; however, the total dose of irradiation to each flask was 10^6 roentgens. The irradiated suspensions were diluted 1:10 in gelatin-saline, plated on SB agar to test for viability, and injected into mice within two hours after irradiation.

C. IMMUNIZATION AND CHALLENGE SCHEDULES

Immunizing injections of 1.0 milliliter containing approximately 10^9 organisms were administered intraperitoneally to 15- to 20-gram white mice. Mice were challenged intraperitoneally two weeks later with approximately 200 SCHU S4 strain organisms suspended in gelatin-saline. Foshay vaccine, prepared by Dr. Lee Foshay, was a whole-cell suspension of strain SCHU S4, concentrated by centrifugation, sterilized by 0.25 per cent phenol and 0.1 per cent thimerosal, and lyophilized. When reconstituted with 0.25 per cent aqueous phenol, the vaccine contained approximately 7.5×10^9 killed organisms per milliliter.

III. RESULTS

Initial experiments were designated to elucidate the interrelationships among the dose of irradiation, cell viability, virulence, and antigenicity. Several concentrations of attenuated LVS organisms and virulent SCHU S4 organisms were exposed to graded doses of X-irradiation in the absence of cysteamine. The results of several experiments that were considered basic to further studies are compiled in Table I.

TABLE I. RELATIONSHIPS AMONG CELL CONCENTRATION, IRRADIATION DOSE, AND IMMUNIZING CAPACITY

Immunizing Strain	X-Ray Dose, roentgens	Viable Cells/ml Before Irradiation	Viable Cells/ml After Irradiation	Mouse Response[a]
LVS	20,000	1.03×10^8	790	Immunized[b]
LVS	30,000	1.03×10^8	0	Not immunized[c]
SCHU S4	30,000	4.0×10^8	320	Death[d]
SCHU S4	40,000	4.0×10^8	0	Not immunized
SCHU S4	300,000	1.67×10^{10}	160	Death
SCHU S4	600,000	1.67×10^{10}	0	Immunized

a. Groups of 10 mice were injected intraperitoneally with 1.0 milliliter of a 1:10 dilution of the irradiated suspensions; survivors were challenged intraperitoneally two weeks later with approximately 100 SCHU S4 organisms.
b. Immunized: Challenged mice demonstrated a higher per cent survival and increased Average Day of Death (ADD) over unimmunized control mice.
c. Not immunized: No differences were observed between experimental and control mice.
d. Death: Mice succumbed during the immunization period.

As the concentration of bacteria was increased, the dose of irradiation required for sterilization increased. This observation is compatible with irradiation studies in which the D_{37}* has been measured.[10] The increased amount of irradiation required to kill bacteria as the cell concentration is increased has been attributed to a decreased oxygen tension resulting from an imbalance between the endogenous respiration of the cells and the diffusion of oxygen into the suspension.[11] Although less than 1 in 10^3 LVS organisms survived 20,000 roentgens, the surviving organisms, even at a 1:10 dilution, induced active immunity (Table I, line 1). Furthermore, irradiated SCHU S4 strain organisms killed mice in low concentrations when less than 1 in 10^8 organisms survived irradiation. These two observations indicated that the immunogenicity and the virulence of the surviving organisms were not appreciably altered by irradiation. The requirement for a high concentration of nonviable *F. tularensis* organisms for immunization

* Dose of irradiation that permits survival of 37 per cent of the organisms.

is also evident in Table I. The results of other experiments on cell concentration revealed that at least 10^7 irradiated organisms were necessary to induce a measurable degree of resistance, neither survival time nor per cent mortality was altered in challenged mice after immunization with less than 10^8 irradiated organisms. The results recorded in the last line of Table I were the first indication that irradiated bacteria could induce active immunity to tularemia.

A. EFFECT OF CYSTEAMINE ON IMMUNOGENIC CAPACITY OF IRRADIATED ORGANISMS

The dose of X-irradiation required to sterilize a saline suspension of 10^{10} bacteria per milliliter is of such magnitude that the indirect effects of radiation due to formation of free radicals are of importance.[12] In an attempt to minimize chemical alterations of antigenic components by free radicals, bacteria were irradiated in the presence of cysteamine. This compound was selected because of its protective effect on bacteria against the indirect effects of ionizing radiation.[13] Suspensions of SCHU S4 strain organisms were exposed to X-rays in the presence of 0.02 M cysteamine to determine the effect on the antigenicity of irradiated cells. An irradiation dose of 10^6 roentgens sterilized all bacterial suspensions. The immunogenicity of irradiated bacteria was greater if cysteamine was present during irradiation (Figure 1); higher concentration of cysteamine appeared to be no more effective than 0.02 M (Table II). The toxic dose of cysteamine for mice was between 4.5 milligrams and 9.0 milligrams.

Seven replicate experiments were carried out in which cells were exposed to 10^6 roentgens in the presence of 0.02 M cysteamine. Groups of 10 to 20 mice were immunized intraperitoneally with 1.0 milliliter of a tenfold dilution of the irradiated vaccine, and challenged two weeks later with approximately 200 SCHU S4 organisms. Table III presents the combined results of these experiments.

B. NONVIABILITY OF IRRADIATED PREPARATIONS

The nonviability of irradiated bacteria has special significance because mice can be effectively immunized with viable attenuated strains of P. tularensis.[8,14,15] Proof that the irradiated bacteria employed in these experiments were nonviable is based on the following observations. No colonies developed when irradiated suspensions were plated on SB agar. If as few as one to ten viable SCHU S4 strain organisms remained in a 1:10 dilution of the irradiated vaccine, mice would have succumbed prior to challenge. This did not occur. Viable P. tularensis could not be isolated from the liver, lung, or spleen of mice sacrificed on Day 1, 2, 3, 6, or 10 following immunization with irradiated suspensions. Furthermore, daily administration of 400 micrograms of streptomycin to mice starting one day before and ending six days after a single immunizing injection of irradiated

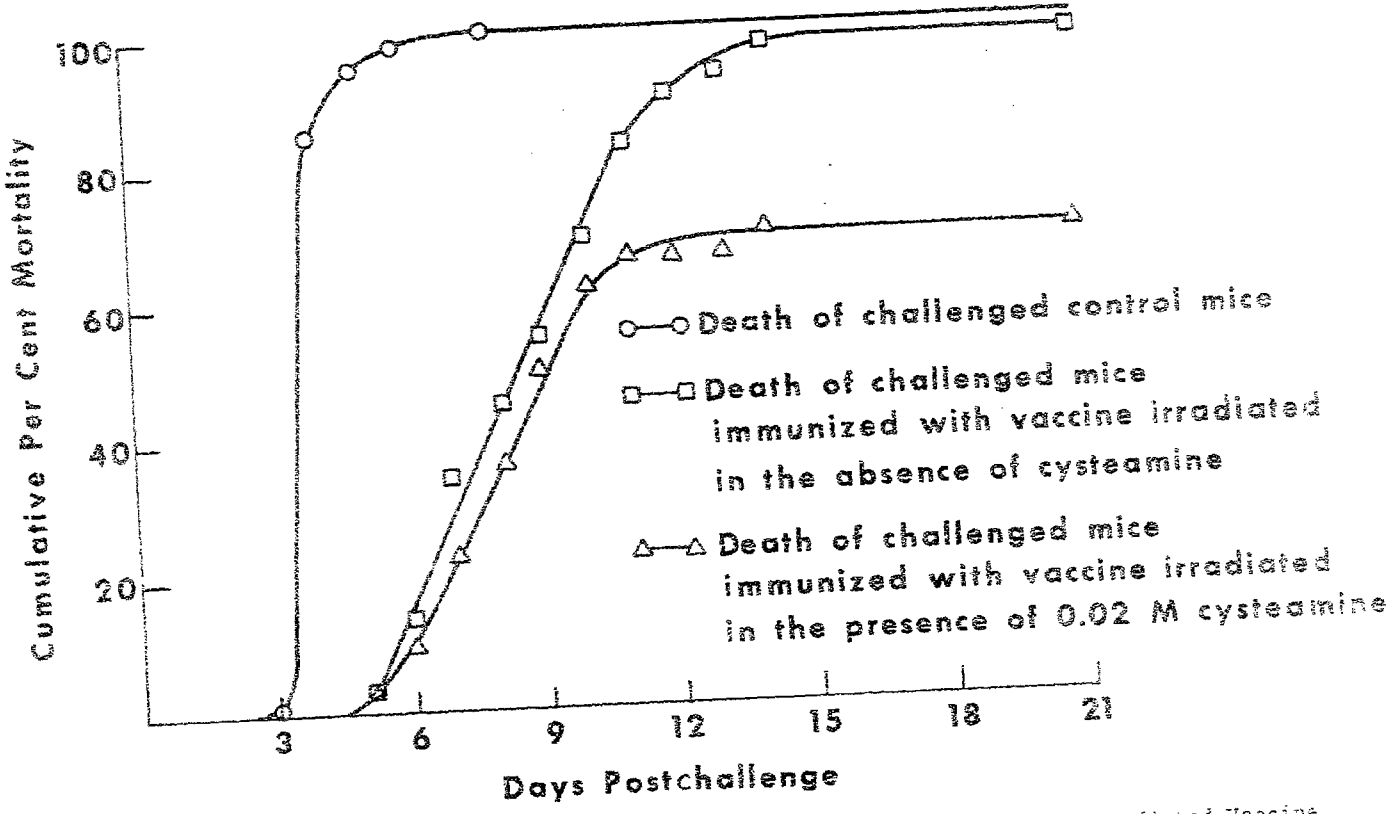

Figure 1. Effect of Cysteamine on the Efficacy of Pasteurella tularensis Irradiated Vaccine.

TABLE II. EFFECT OF CYSTEAMINE CONCENTRATION ON ANTIGENICITY

Cysteamine Concentration[a]	Mortality Ratio[b]	Mortality,%	Average Day of Death
0.02 M	8/10	80	11.1
0.05 M	10/10	100	8.8
0.10 M	7/8	88	10.6
0.20 M	16/19	84	11.4
0.80 M	Toxic for mice		
Controls: No immunization	10/10	100	3.9

a. Cysteamine concentration during irradiation. Preparations were diluted 1:10 prior to injection.
b. Number of mice succumbing to challenge. / Number of mice challenged.

TABLE III. IMMUNOGENICITY OF IRRADIATED VACCINES[a]

	Mortality Ratio[b]	Mortality,%	Average Day of Death
Irradiated vaccine	71/89	80 (50-95)[c]	11.8 (9.9 - 15.2)
None	68/68	100	4.4 (4.0 - 4.7)

a. Combined results of seven replicate experiments.
b. Number of mice succumbing to challenge. / Number of mice challenged.
c. Numbers in parentheses refer to range of individual experiments.

organisms did not interfere with the development of immunity. Such streptomycin treatment has been shown to inhibit the development of immunity that normally follows administration of viable attenuated organisms.*

A vaccine preparation that contained no preservative retained immunizing activity for seven days at 5°C, but immunogenicity was lost by the fiftieth day of storage (Table IV).

TABLE IV. EFFECT OF STORAGE ON IMMUNOGENICITY OF IRRADIATED VACCINE[a]

Time of Storage at 5°C	Per Cent Mortality	Average Day of Death
2 hours	80	12.9
1 day	89	10.0
7 days	70	12.6
50 days	100	8.7
Control, no immunization	100	4.2

a. Vaccine prepared by irradiation with 10^6 roentgens in the presence of 0.02 M cysteamine. Mice were immunized with 1.0 milliliter intraperitoneally containing approximately 1.5×10^9 organisms and challenged two weeks later with SCHU S4 strain organisms.

* Gordon, M., U.S. Army Biological Laboratories: unpublished results.

C. EFFECTIVENESS OF CHEMICALLY KILLED AND VIABLE VACCINES

The immunogenicity of two other types of P. tularensis vaccines were evaluated. The Foshay vaccine contained approximately 7.5×10^9 killed SCHU S4 strain organisms per milliliter. Viable vaccine was prepared from a 24-hour-grown SB agar slant of LVS organisms. Cells were removed from the slant with gelatin-saline and diluted to contain 227 organisms per milliliter. Groups of 36 to 39 mice were injected with 1.0 milliliter intraperitoneally of one of the vaccine preparations. Fourteen days later each of the groups was divided into four subgroups and challenged intraperitoneally with 20,000, 2,000, 200, or 20 viable SCHU S4 strain organisms. Mice injected with the Foshay vaccine were not protected against an intraperitoneal challenge with even small challenge doses; one animal of ten survived challenge with 20 SCHU S4 strain organisms. These results agree with previous results of Ruchman and Foshay[5] with an almost similar phenol-killed vaccine. All animals injected with viable vaccine survived challenge with from 20 to 20,000 SCHU S4 strain organisms. These results agree with those reported by Eigelsbach and Downs.[6]

IV. DISCUSSION

Evaluation of the immunizing activity of the irradiated vaccine preparation requires comparison with previous nonviable vaccines and with attenuated viable vaccines. The antigenicity of previous nonviable vaccines was demonstrated in animals less susceptible to tularemia than white mice[3] by challenge with strains of less than full virulence,[1] or with extremely small challenge doses.[2,5] These vaccines usually failed to immunize susceptible animals to the degree that they could survive challenge with appreciable numbers of fully virulent organisms. Results obtained with the vaccine of Foshay in the present study were in agreement with those in previous reports.[5]

The ether-extracted vaccine described by Bell and colleagues has been evaluated almost entirely by challenge of immunized animals with strain 425 of P. tularensis.[1] Although this strain is highly virulent for mice, it is essentially avirulent for rabbits; mice are readily protected against it by various nonviable antigens. The demonstration of immunization against this strain by the ether-extracted vaccines provides no indication that the vaccine would also protect against fully virulent strains such as SCHU S4. In limited experiments, three injections of the vaccine were shown to confer detectable immunity against subcutaneous challenge with strain 14, stated to be fully virulent.[1] It is probable that ether-extracted vaccine had greater protective activity than Foshay vaccines.

Evidently the immunity against challenge with fully virulent organisms that is evoked by the present irradiated preparations is of a higher order than that evoked by Foshay vaccine. The immunity may also be greater than that evoked by the ether-extracted vaccine of Bell and associates, although no direct comparison has been made. The present irradiated preparations are less effective than viable vaccines, however, at least in the immunization of mice.[9] Many variables that may influence the antigenicity of the irradiated preparations have not been investigated, and it seems probable that considerable increase in antigenicity may be anticipated as a result of further study. Doubtless the protection may also be increased by use of multiple immunizing injections and by administration of the challenge dose by routes that represent a less severe test of immunity than the intraperitoneal.

Ionizing radiation has been shown to be a superior killing agent in preparation of other vaccines. Polley[16] demonstrated the induction of active immunity with gamma-irradiated influenza virus. It was reported by Carpenter et al[17] that irradiated tubercle bacilli were as effective as BCG vaccine in protecting mice against tuberculosis. Donaldson and Mitchell[6] showed that the administration of X-irradiated preparations of Ehrlich's ascites carcinoma protected mice against a subsequent transplant of this tumor, whereas preparations killed by desiccation, freezing and thawing, or by mechanical grinding were ineffective.[18]

IV. SUMMARY

Pasteurella tularensis strain SCHU S4 rendered nonviable by exposure to X-irradiation was shown to immunize mice as determined by increased resistance to intraperitoneal challenge with several hundred virulent organisms. The mean survival time of the immunized animals was increased markedly as compared with controls and a portion, ranging from 5 to 50 per cent in different experiments, survived challenge. Injection of approximately 10^8 organisms was necessary to stimulate a minimal immune response. The antigenicity was increased when 0.02 M cysteamine was added to the bacterial suspension prior to irradiation to minimize the indirect effects of irradiation. Nonviability of the irradiated vaccines was established by culture, by repeated failure to detect viable organisms in tissues of immunized mice, and by persistence of the protective effect of the vaccine when the animals received streptomycin for several days following immunization.

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